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NETRIN and SLIT guide salivary gland migration

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Abstract

Directed migration is pivotal for the proper placement and function of nearly all organs. The majority of known guidance molecules involved in directed migration have been identified from studies of migrating axons during nervous system development. Here, we show that at least two of these axon guidance molecules, NETRIN and SLIT, act through their canonical receptors, to guide *Drosophila* embryonic salivary glands. NETRIN serves as a chemo-attractant while SLIT functions antagonistically to NETRIN as a chemo-repellent during salivary gland migration. CNS midline expression of both NETRIN and SLIT directs the glands to move unswervingly parallel to the CNS. NETRIN expression is also required in the visceral mesoderm, along which the glands move during their migration. We propose that analogous to axon guidance, a balance between chemo-attractants and chemo-repellents is required for the proper migratory path of the developing salivary glands.
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Keywords: Salivary gland; Guidance cues; NETRIN; SLIT; Integrins; Migration; Organogenesis

Introduction

Chemotactic cell migration plays a central role during development and throughout life. Failure of cell or tissue migration can result in disease or organismal death. Some forms of mental retardation have been attributed to defects in neuronal migration, while arthritis, osteoporosis, muscular dystrophy, and asthma are all associated with abnormal leukocyte migrations (D'Ambrosio et al., 2003). Cardia Bifida, an embryonic lethal developmental disorder that results in two "hearts", is due to a failure of convergent migration by the left and right cardiac primordia during embryogenesis (Compernolle et al., 2003; Saga et al., 1999). Less severe tissue migration disorders such as salivary gland ectopias that are a result of misplaced salivary gland tissue in the middle ear, can cause deafness (Ookouchi et al., 2003; Supiyaphun et al., 2000). To reach their appropriate destinations, both individual cells and multicellular tissues rely on signaling molecules for guidance. Unlike individual cells that respond to guidance cues independently, multicellular tissues must often move as a coherent group. This concerted cell migration not only results in the proper positioning of tissues such as the kidney, lung, and salivary glands, but also sculpts their final three-dimensional appearance. Ultimately, and most importantly, understanding the guidance mechanisms used by migrating cells and tissues could lead to the creation of novel therapeutic approaches to treat cell migration disorders.

The *Drosophila* embryonic salivary gland serves as a helpful model to further our knowledge of tissue migration. It is a simple, unbranched, tubular tissue that undergoes a stereotyped migration. Salivary glands begin as a pair of single-layered epithelial disks, called salivary placodes, that invaginate via apical constriction to form slender tubes (Figs. 1A,B). As they leave the surface of the embryo, these tubes extend dorsally and posteriorly at a 45° angle on either side of the central nervous system until they reach the visceral mesoderm (Figs. 1C,D). At this point in their migration, they change paths and begin to move horizontally along the mesoderm (Figs. 1E,F) until they lie horizontally inside the embryo, dorsal and lateral to the CNS (Figs. 1G,H).

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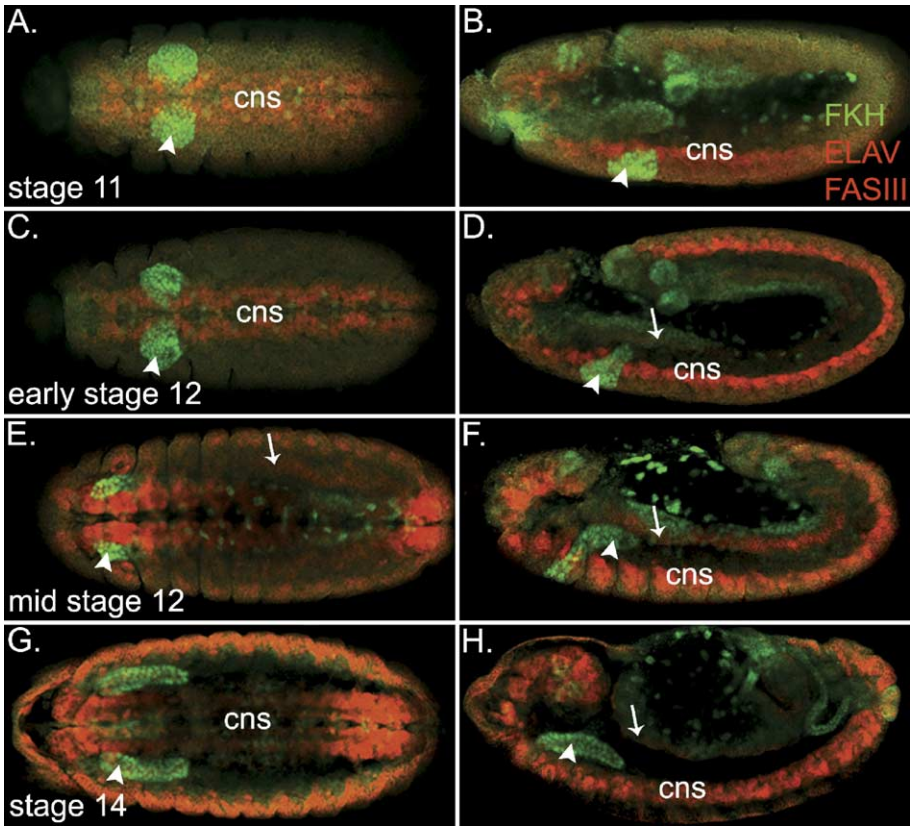

Fig. 1. Structure of the developing salivary glands and their proximity to the CNS and visceral mesoderm. Salivary gland cells (arrowhead) are stained for FKH in green, the CNS is stained for ELAV in red, and the visceral mesoderm (arrow) is visualized by FASIII staining, also in red. (A, C, E, G) Ventral and (B, D, F, H) the corresponding lateral views of developing embryos from stages 11 through 14. (A, B) At stage 11, cells of the salivary gland are on the surface of the embryo and those closest to the midline are directly ventral to the developing CNS. (C, D) During early stage 12, salivary gland cells invaginate dorsally towards the interior of the embryos at a 45° angle. (E, F) Upon reaching the visceral mesoderm, salivary glands migrate posteriorly along it throughout stages 12 and 13. (G, H) At stage 14, the salivary glands reach their final resting position and lie dorsal and lateral to the central nervous system (CNS).

Since salivary glands undergo directed migration, orientation signals might guide the developing organs to their proper positions. Such guidance signals can come in the form of diffusible chemo-attractants and chemo-repellents as well as through cell–cell interactions. To date, the guidance cues required to instruct the gland cells to move in a particular direction have not been identified. Only integrins, cell surface receptors involved in adhesion to the extracellular matrix, have been implicated in salivary gland migration. Integrin αPS2 (INFLATED, INF) is required in the visceral mesoderm and αPS1 (MULTIPLE EDEMATOUS WINGS, MEW) in the salivary gland for the migration of the gland along the visceral mesoderm (Bradley et al., 2003).

Since numerous chemo-attractants and chemo-repellents that direct axon guidance have been identified, we explored the possibility that these same molecules might also direct salivary gland migration. Here, we show that two guidance cues, NETRIN and SLIT, work in opposition to each other for proper salivary gland migration. Although SLIT has established functions in both non-neural and neural tissues, this is the first demonstration of a chemo-attractant function for *Drosophila* NETRIN outside the nervous system.

Materials and methods

Fly strains

The following deficiencies and alleles were used: *NP5* deficiency spanning both the *netA* and *netB* genes, fra^{4}, fra^{3}, $robo1^{GA285}$, $robo1^{GA285}robo2^{1L135}$, $robo3^{1}$ (all provided by C. Goodman) (Kolodziej et al., 1996; Mitchell et al., 1996; Rajagopalan et al., 2000a,b), $robo2^{1}$, $robo1^{1}$ $robo2^{1}$ (both provided by B. Dickson), $comm^{E39}$ (provided by G. Tear), weg^{M454} (provided by C. Klämbt) (Hummel et al., 1999a), mew^{M6} and $slit^{2}$ (both from the Bloomington Stock Center).

The following GAL4, UAS, and transgenic fusion strains were used: *UAS-netA*, *UAS-netB*, *UAS-fra*, *UAS-unc-5*, *slit:netA*, *slit:netB* (all provided by B. Dickson) (Mitchell et al., 1996), *sca-GAL4* (provided by M. Foss), *twi-GAL4* (provided by M. Baylies), *prd-GAL4*, *bap-GAL4* (Weiss et al., 2001), *UAS-slit* (provided by C. Samakovlis), *UAS-lacZ* (Brand and Perrimon, 1993). Although *prd-GAL4* is expressed early in a pair-rule gene pattern, its expression continues in parasegment 2 from which the salivary glands develop.

Immunocytochemistry and in situ hybridization

Embryo fixation and staining were performed as described (Chandrasekaran and Beckendorf, 2003). The salivary gland lumen-specific antibody used was mouse anti-CRUMBS (Cq4) (Developmental Studies Hybridoma Bank, University of Iowa) at 1:25 and the salivary gland nuclear-specific antibody used was rabbit anti-FKH (1:1000). Rabbit anti β-galactosidase was also used at 1:1000 (Vector laboratories, CA). The mouse anti-SLIT (C555.6D), mouse anti-ROBO1 (13C9), mouse anti-ROBO3 (15H2), and mouse anti-FASIII (7G10) antibodies were all obtained from the Hybridoma Bank and used at 1:10. Rabbit antiserum against ROBO2 (from B. Dickson) (Rajagopalan et al., 2000a) was used at 1:2000. The appropriate biotinylated secondary antibodies were used at 1:200 together with the Vector Elite ABC kit (Vector Laboratories, CA) and photographed using the Nomarski optics on the Leica DMRB microscope. Alexa Fluor-546- and 488- (Molecular Probes) secondary antibodies were used at 1:500 and visualized by the Zeiss 510 confocal microscope. Whole-mount in situ hybridization was performed as described (Tautz and Pfeifle, 1989) with modifications (Harland, 1991) using antisense digoxigenin-labeled probes. BCIP and nitro blue tetrazolium were used as substrates for alkaline phosphatase to visualize the signal. After being rinsed, cleared with 50% glycerol and then 70% glycerol, embryos were visualized and photographed using Nomarski optics on the Leica DMRB microscope.

Results

Embryos deficient for both netA and netB exhibit salivary gland guidance defects

The *Drosophila* genome includes two functionally redundant *netrin* genes, *netA* and *netB* (Harris et al., 1996; Mitchell et al., 1996). Mutant embryos carrying the deficiency *Df(1)NP5*, that removes both *netrin* genes, show gland guidance defects. Instead of the glands lying parallel to the midline, 23% of *netrin*-mutant embryos have glands that curve laterally, away from the midline, while 9% have glands that curve medially, towards the midline (Fig. 2B). The initial invagination of the salivary gland in *Df(1)NP5* mutant embryos appears normal, and it is only after the gland has reached the visceral mesoderm that it becomes laterally misrouted (data not shown). Immunohistochemistry performed with FASCICLIN III (FASIII), a visceral mesoderm marker, did not reveal any defects in the structure/integrity of the visceral mesoderm. In addition, gut constrictions appear normal in *netrin* mutants, suggesting that this function of the visceral mesoderm is unaffected. Since *Df(1)NP5* embryos have thinner than normal or completely absent commissures in the ventral nerve cord, we investigated whether the commissural defects are

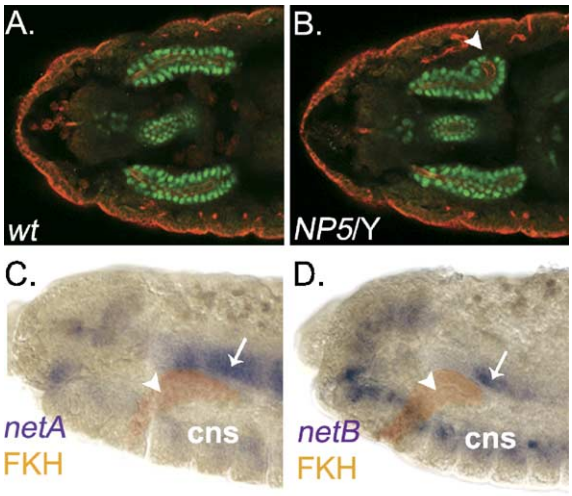

Fig. 2. *netrin*-mutant phenotype and expression. (A, B) NETRINS are needed for proper salivary gland guidance. Ventral views of stage 15 embryos. Salivary glands are visualized with the nuclear marker FKH, in green, and the apical marker CRB, in red. *NP5* mutant embryos lack both *netA* and *netB*; most of the misguided glands become oriented laterally (B, arrowhead) instead of extending posteriorly as seen in wild type (A). (C, D) Proximity of the salivary gland to *netrin*-expressing tissues. Lateral views of stage 12 embryos. *netA* (C) and *netB* (D) RNA expression is evident in both the visceral mesoderm (arrow) and in the midline glia of the central nervous system (CNS). Throughout its migration, the salivary gland (arrowhead), stained with anti-FKH in orange, comes in close proximity to *netA*- and *netB*-expressing tissues.

connected with salivary gland defects. Using monoclonal antibody BP102 to visualize commissures and FORK HEAD (FKH) antibody to visualize the salivary glands, we were able to determine that commissure abnormalities seen in *netrin* mutants do not correlate with salivary gland defects. In addition, *weniger* mutants that have comparable commissural defects as those seen in *netrin*-mutant embryos (Hummel et al., 1999a,b) lack salivary gland defects (data not shown). Similarly, *commissureless* mutants that have much more severe commissural defects than either *netrin* or *weniger* (Tear et al., 1996) also lack salivary gland abnormalities (data not shown). These experiments together demonstrate that the misrouting of the glands observed in *Df(1)NP5* embryos are not due to defects of the visceral mesoderm or CNS, but instead result from a lack of NETRIN guidance during salivary gland development.

NETRIN is needed in both midline cells and the visceral mesoderm for salivary gland guidance

To establish which tissue(s) must express NETRIN for proper salivary gland guidance, we examined *netA* and *netB* RNA expression. During salivary gland migration, both *netA* and *netB* are expressed in the midline glia of the CNS and in the visceral mesoderm (Harris et al., 1996; Mitchell et al., 1996 and Figs. 2C,D). Invaginating salivary glands begin their migration in close proximity to the *netrin*-expressing midline cells and later come in contact

with the *netrin*-expressing visceral mesoderm. To determine whether NETRIN is required in the visceral mesoderm for salivary gland guidance, we utilized the *GAL4-UAS* system (Brand and Perrimon, 1993) to express NETRIN in the visceral mesoderm of *netrin* mutants. Embryos mutant for *Df(1)NP5* carrying both *bap-GAL4* and *UAS-netB* were analyzed and found to have completely rescued gland guidance defects (Table 1).

Despite the successful rescue by visceral mesoderm expression, we also tested whether NETRIN expressed only at the midline can rescue the misguided glands. We analyzed the phenotype of *Df(1)NP5* embryos that carried one copy of a *netA* or *netB* transgene driven by the *slit* promoter (Mitchell et al., 1996). Expressing either *netA* or *netB* in the midline of *netrin* mutants partially rescued the gland guidance defects. In contrast to the 32% gland guidance defects seen in *netrinA/B* mutant embryos, only 4–6% of the rescued embryos have gland defects (Table 1). Therefore, NETRIN in both the visceral mesoderm and in the midline glia are involved in guiding the salivary glands during their development. The complete visceral mesoderm rescue might be due either to a greater role of visceral mesoderm NETRIN compared to midline NETRIN during gland migration or to different strengths of the GAL4 drivers used for the rescue experiments. Since the majority of misguided salivary glands in *Df(1)NP5* embryos curve away from the midline and off of the visceral mesoderm, NETRIN appears to serve as an attractant during gland guidance.

Ectopic NETRIN attracts salivary glands to its sites of expression

If NETRIN functions as an attractant during gland migration, it should be possible to misdirect the glands towards the source of ectopic NETRIN expressed in tissues other than the visceral mesoderm or midline glia. When *netB* is expressed throughout the mesoderm (in an otherwise wild-type embryo) using the *twist*-GAL4 driver, 16% (n = 51) of the embryos have salivary glands that split into two paths, one following *netrin* in the visceral mesoderm and the other following *netrin* in the somatic mesoderm (Fig. 3E). Similarly, when *netB* is expressed throughout the entire developing CNS with *sca*-GAL4, 63% (n = 49) of the embryos possess glands that become oriented toward the ectopic source of *netrin* (Fig. 3B). The weaker pan-neural *elav*-GAL4 driver was not sufficient to cause misorientation of the glands. We saw no effect on gland guidance when *netA* was ectopically expressed in the same tissues. The differences between *netA* and *netB* are most likely due to the lower level of expression from the UAS-*netA* line (Mitchell et al., 1996), but we cannot exclude the possibility that the two NETRIN proteins may have slightly different roles in gland guidance. However, based on the misexpression experiments, NETB is a strong chemo-attractant sufficient to guide an entire developing tissue.

Table 1
Penetrance and rescue of gland guidance defects

% of embryos with gland guidance defects			
Genotype	Glands curve laterally (%)	Glands curve medially (%)	Number of embryos scored
Df(1)*NP5*/Y	23	9	111
Df(1)*NP5*/Y;*slit:A*/+	3	3	95
Df(1)*NP5*/Y;*slit:B*/+	2	2	66
Df(1)*NP5*/Y; *bap*-GAL4/UAS-*netB*	0	0	53
W^{1118}	0	0	120

slit:A = *netA* driven by the *slit* promoter.
slit:B = *netB* driven by the *slit* promoter.

Embryos deficient for frazzled have salivary gland guidance defects similar to netrin-mutant embryos

Two known NETRIN receptors exist in *Drosophila melanogaster*, the DCC family receptor FRAZZLED (FRA) and UNC-5 (Keleman and Dickson, 2001; Kolodziej et al., 1996). Studies performed in both *Drosophila* and *Xenopus* have shown that attraction to NETRIN involves multimerization of DCC/FRA while repulsion can be mediated either through the interaction between DCC/FRA and UNC-5 or through UNC-5 alone (Hong et al., 1999; Keleman and Dickson, 2001; Stein et al., 2001). Since NETRIN serves as an attractive guidance cue during gland migration, we hypothesized its effect to be mediated through the attractive NETRIN receptor, FRAZZLED. To investigate the role of DCC/FRA in gland guidance, we examined fra^3 and fra^4 null mutants. Since fra^3 and fra^4 EMS alleles were obtained in two different backgrounds (Koloziej et al., 1996), we checked for gland guidance defects in trans-heterozygous fra^3/fra^4 mutant embryos. In *fra* mutants, partially penetrant gland guidance defects are observed; 4% of the mutant embryos have glands that curve laterally (Fig. 4B) and 2% have glands that curve medially (Table 2). The gland guidance defects in *fra* mutants strongly resemble those observed in *netrinA/B* mutant embryos suggesting that at least part of NETRIN's chemo-attractive effect is mediated through the FRA receptor. Consistent with its apparent role in gland guidance, immunohistochemistry showed that FRA protein is expressed in invaginating salivary gland cells (Fig. 4E).

The low penetrance of gland guidance defects in *fra* mutants, however, suggests that a second NETRIN receptor may act redundantly with FRA during salivary gland migration. Since NETRIN has been shown to serve as a ligand for integrins during fetal pancreatic development (Yebra et al., 2003), we examined whether this might also apply to gland development. Integrins are transmembrane heterodimers consisting of α and β subunits that link the cytoskeleton of the cell to the ECM and consequently transmit forces and signals necessary for migration (review by Bokel and Brown, 2002). Of the five α and two β

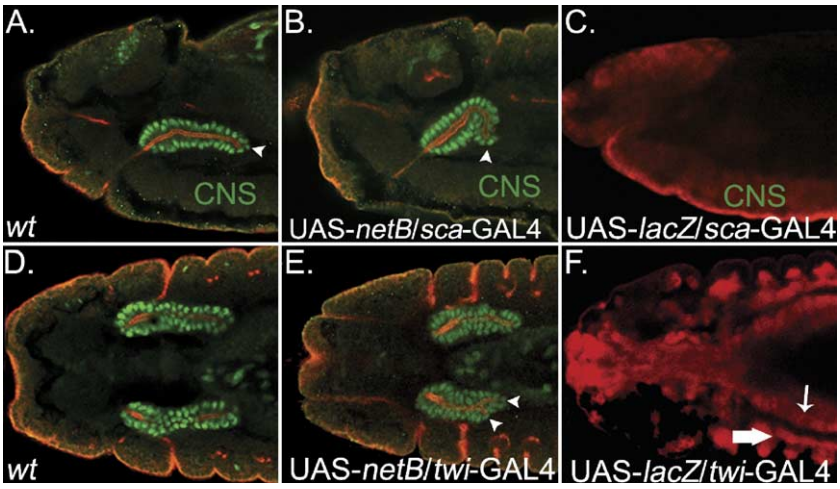

Fig. 3. Salivary glands are attracted toward sites of ectopic *netrinB* expression. (A, B, D, E) Salivary glands are visualized by FKH antibody in green and anti-CRB in red. (C, F) The ectopic sites at which *netrin* is expressed are visualized by anti-β-GAL. (A–C) Lateral views of stage 14 embryos. When *netrinB* is overexpressed throughout the entire CNS with the *sca*-GAL4 driver, the distal tips of glands become curved toward the CNS (B, arrowhead), instead of lying parallel to it (A, arrowhead). The CNS pattern of expression produced by the *sca*-GAL4 driver when crossed to UAS-*lacZ* flies (C). (D–F) Ventral views of stage 14 embryos. Ectopic expression of *netB* throughout the somatic and visceral mesoderm with *twi*-GAL4 causes the distal tip of the gland to split into branches that migrate in different directions (E). As shown clearly by the CRB staining of the lumen (red), one branch of the gland continues to move along the visceral mesoderm, while the other appears to migrate toward lateral somatic muscles (arrowheads). The expression pattern created by the *twi*-GAL4 driver when crossed with UAS-*lacZ* flies (F). The visceral mesoderm is indicated by a thin arrow and the lateral somatic muscle by a thick arrow.

subunits encoded by the *Drosophila* genome, only αPS1 (MEW) and βPS (MYS) are expressed in the gland and play a role in gland migration (Bradley et al., 2003). By reexamining gland defects in *mew* mutants, we determined that integrin αPS1/βPS most likely does not affect gland migration by serving as a second NETRIN receptor. While *mew* mutants have curved glands as seen in *netrin* and *fra*, they are much more penetrant and lack a lateral/medial bias. 35% of *mew* mutant embryos have at least one gland that curves medially while another 35% of the embryos have at least one gland that curves laterally (Table 2). Instead of moving along the visceral mesoderm, the leading tip of the *mew* salivary gland loses adhesion to the visceral mesoderm and falls off laterally or medially on either side of it with

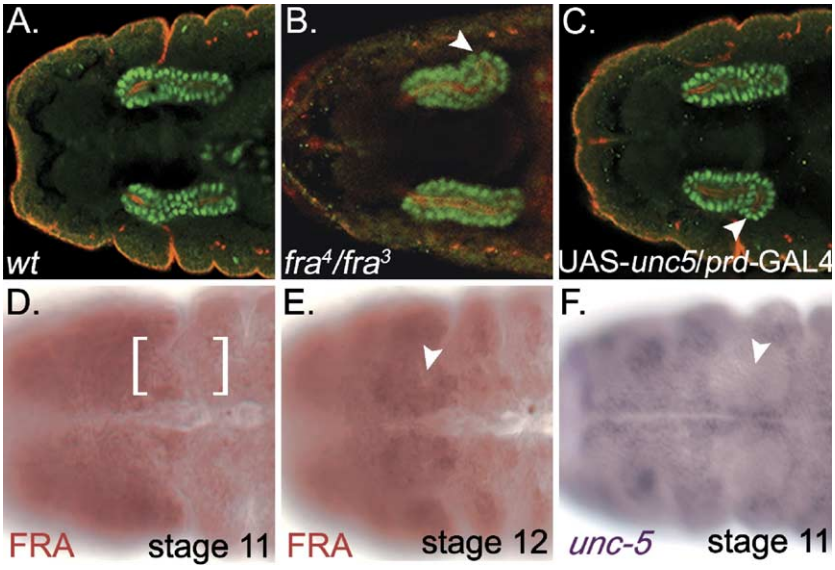

Fig. 4. Expression of the *netrin* receptors, *frazzled* and *unc-5*, and their role in salivary gland guidance. (A–C) Ventral views of embryos stained with FKH antibody in green and CRB antibody in red. In fra^4/fra^3 transheterozygous mutant embryos, the majority of misrouted glands curve laterally, closely resembling the *netrin*-mutant gland phenotype (compare panel B to Fig. 2B). Correspondingly, FRA protein is present throughout the ventral epidermis prior to salivary gland invagination at stage 11 (D, region of salivary placode is between brackets) and becomes more strongly expressed in salivary gland cells during their invagination at stage 12 (F, site of gland invagination is designated by an arrowhead). Conversely, the repulsive netrin receptor, *unc-5*, is specifically excluded from the salivary placode (G, arrowhead). Misexpression of *unc-5* in salivary glands results in laterally curved glands (C, arrowhead).

Table 2
Penetrance and *mew*, *fra* genetic interactions

% of embryos with gland guidance defects			
Genotype	Glands curve laterally (%)	Glands curve medially (%)	Number of embryos scored
mew^{M6}/Y	35	35	57
mew^{M6}/Y; fra^{4}/+	50	16	74
fra^{4}/fra^{3}	4	2	114
mew^{M6}/Y; UAS-*fra*/+; *prd*-GAL4/+	16	50	60
W^{1118}	0	0	120

equal probability (TK, unpublished observations). If integrin αPS1/βPS served as an attractive NETRIN receptor, the majority of mutant embryos would be expected to have glands that curve laterally as is seen in both NETRIN and FRA mutants. Although MEW does not appear to behave jointly with FRA as a NETRIN receptor, the *fra* mutation does bias the *mew* salivary gland phenotype. Although misguided glands in *mew* mutants have an equal chance of lateral or medial displacement, when these embryos are also heterozygous for *fra*, three quarters of the misguided glands curve laterally (Table 2). Conversely, using the *prd*-GAL4 driver to overexpress FRA in the glands of *mew* mutants causes three quarters of the misguided glands to curve medially (Table 2). These results indicate that loss of adhesion between the salivary gland and the visceral mesoderm in *mew* mutants makes the gland more sensitive to midline cues. In this sensitized background, increasing the levels of FRA in the gland enhances the gland's attraction toward NETRIN at the midline, further supporting the role of FRA as a NETRIN receptor during gland migration.

Since the chemo-attractive effect of NETRIN on gland guidance is mediated through the FRA receptor expressed in the gland, the repulsive NETRIN receptor, UNC-5, was not expected to be involved. We found that throughout salivary gland development, *unc-5* RNA is specifically excluded from the salivary placodes and the invaginating salivary gland cells (Fig. 4F and data not shown). Ectopically expressing *unc-5* in the glands of an otherwise wild-type embryo, however, caused gland guidance defects. When flies containing the salivary gland *prd*-GAL4 driver were crossed to those carrying the UAS-*unc-5* transgene, 70% (n = 65) of the embryos had laterally oriented salivary glands that appear to be repelled by the CNS and visceral mesoderm (Fig. 4C). This phenotype suggests that the normal chemo-attractive function of NETRIN during salivary gland migration can be manipulated by changing the receptor responding to it.

SLIT signaling is also required for salivary gland guidance

The lateral deflection of the salivary glands in *netrin* mutants might be caused by a centrally located repellent. A possible candidate for such a repellent is SLIT, a large extracellular matrix protein made and secreted by the midline glia (Rothberg et al., 1990). Initially identified as a chemo-repellent involved in guiding axons during nervous system development, it has since been implicated in the guidance of both somatic muscle and the trachea (Englund et al., 2002; Kramer et al., 2001). Here, we show that SLIT is also required for salivary gland guidance. Instead of the glands lying parallel to the midline, as in wild-type embryos, 90% (n = 55) of *slit*-mutant embryos exhibit glands that curve medially, towards the midline (compare Figs. 5A and 5B). The *slit*-mutant glands may become oriented towards the midline by their strong attraction to high levels of NETRIN, and possibly other attractants expressed there.

To further establish the role of SLIT as a repellent during gland development, we misexpressed it in the visceral mesoderm during salivary gland migration. When *slit* is expressed in the visceral mesoderm with the *bap*-GAL4 driver (in a wild-type background), the glands curve ventrally, away from the visceral mesoderm (Fig. 5D). Therefore, ectopic expression of *slit* corroborates its role as a chemo-repellent in salivary gland guidance.

SLIT signaling is mediated through ROUNDABOUT (ROBO) receptors (Kidd et al., 1999). The *Drosophila* genome encodes three of these: ROBO1, ROBO2, and ROBO3. Immunohistochemistry shows that ROBO1 and ROBO2 are expressed in salivary gland cells (Figs. 6C,E) while ROBO3 is excluded (data not shown). To determine if ROBO receptors are required for gland guidance, we examined *robo1* and *robo2* single mutant embryos, and

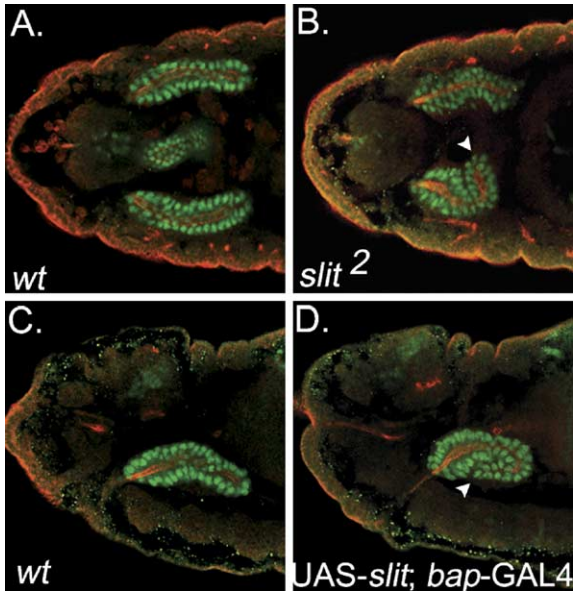

Fig. 5. SLIT repels salivary glands. (A–D) Salivary glands are visualized by FKH antibody in green and CRB antibody in red. (A, B) Ventral views of stage 15 embryos. Salivary glands of *slit*-mutant embryos curve medial-ventrally, towards the CNS (B, arrowhead). (C, D) Lateral views of stage 14 embryos. SLIT misexpression throughout the visceral mesoderm causes glands to curve ventrally, away from the visceral mesoderm (D, arrowhead).

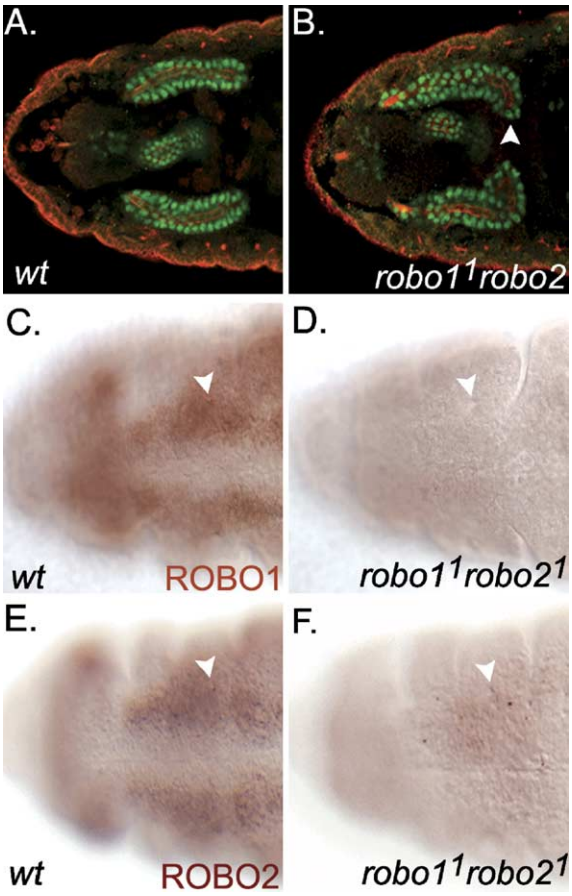

Fig. 6. Expression and mutant phenotype of SLIT receptors, ROBO1 and ROBO2. (A, B) Ventral views of embryos stained with anti-FKH in green, anti-CRB in red. *robo1 robo2* double mutant embryos possess medially curved glands, resembling the *slit* phenotype (compare panel B, arrowhead, to Fig. 5B). (C–D) Ventral views of early stage 12 embryos. Site of salivary gland invagination is indicated by an arrowhead. ROBO1 (C) and ROBO2 (D) are expressed in wild-type salivary gland cells and are absent in *robo1 robo2* mutant embryos (D, F).

robo1 robo2 doubles. While the single mutant embryos have weak gland guidance defects, *robo1 robo2* double mutants have severe guidance defects comparable to those seen in slit (compare Figs. 5B and 6B). This result agrees with axon guidance and somatic mesoderm guidance studies in which *robo1 robo2* double mutants resemble the slit-mutant phenotype and give more severe guidance defects than either of the single mutants alone (Kramer et al., 2001; Simpson et al., 2000).

netrin slit double mutants resemble the slit phenotype

Whereas SLIT appears to be either the strongest or the only chemo-repellent that guides salivary glands, the low penetrance of gland guidance defects in *netrinA,B* mutants suggests that other chemo-attractant(s) at the CNS may act redundantly with NETRIN. If so, then in the absence of both NETRIN and SLIT, the glands would be expected to curve medially, toward these unidentified attractants at the CNS. On the other hand, if NETRINS were the only attractants that function to guide salivary glands, then in the *netrin slit* double mutant, the medial sources of attraction and repulsion would both be absent, and the glands would be expected to migrate in approximately the correct posterior direction. We found that 50% of the double mutants had glands that curve medially, toward the CNS. This phenotype resembles the *slit*-mutant phenotype and suggests the presence of additional midline chemo-attractants that contribute to salivary gland guidance. In addition, since the gland guidance defects in the *netrin slit* double mutants are less penetrant than in the *slit* single mutants (50% vs. 90%), it seems that normally NETRIN and SLIT work in opposition to each other to keep the gland moving parallel to the CNS.

Discussion

Salivary gland migration can be separated into two phases. During the first phase, salivary glands invaginate via apical constriction dorsally into the embryo at a 45° angle until they reach the visceral mesoderm. Subsequently, during the second phase, the salivary glands migrate along the visceral mesoderm until they reach the middle of the third thoracic segment. While it is not yet certain whether extrinsic guidance cues are necessary for the initial migration phase, some properties intrinsic to the salivary gland have been shown to be important. RhoGEF2 and the transcription factor FORK HEAD regulate apical constriction of salivary gland cells during their invagination (Myat and Andrew, 2000b, Nikolaidou and Barrett, 2004; Weigel et al., 1989), while mutations in *huckebein* and *faint sausage* affect the site of invagination (Myat and Andrew, 2000a). Embryos mutant for any of these genes lack the typical 45° invagination, and consequently, the glands do not extend toward the visceral mesoderm. All these genes are expressed within the salivary gland cells. So far, there is no evidence for extrinsic guidance signals during this initial phase of migration.

Here, we show that two guidance cues, NETRIN and SLIT, are required for the second phase of salivary gland migration, when the glands move along the visceral mesoderm. As the glands migrate along the surface of the visceral mesoderm, they are positioned dorsal and lateral to the CNS (Fig. 7). NETRIN expressed by the visceral mesoderm and the CNS midline functions as an attractant to keep the gland moving on a straight path, parallel to the CNS. In the absence of NETRIN or its receptor FRA, the glands lose their course along the visceral mesoderm and become misrouted. If this were merely due to the loss of salivary gland traction along the NETRIN-expressing visceral mesoderm, the glands might fall off either side of the visceral mesoderm with equal likelihood. However, while some glands do curve medially, most of the misguided salivary glands in *netrin* and *fra* mutants curve laterally (Fig. 7). This bias is due to the presence of the midline

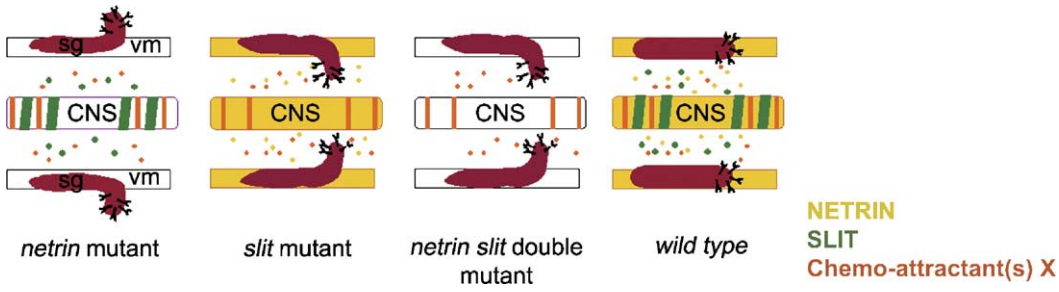

Fig. 7. A schematic summarizing the salivary gland defects of guidance cue mutants. In a *netrin* mutant, salivary glands curve laterally (away from the CNS, and off of the visceral mesoderm) due to the presence of the repellent, SLIT, at the CNS. Salivary gland repulsion by SLIT appears to be greater than the attraction of the yet unidentified attractant (chemo-attractant X). Conversely, in the absence of SLIT, attraction is not balanced by repulsion and consequently, the glands curve medially (toward the CNS) in the direction of high chemo-attractant levels (NETRIN and chemo-attractant X). Due to the persistence of unidentified chemo-attractants in *netrin slit* double mutants, the glands become oriented toward the CNS. In wild-type embryos, a balance between attraction and repulsion exists and the glands are maintained on their proper, posterior migratory path. (Visceral mesoderm, vm; central nervous system, CNS; salivary gland, sg.)

chemo-repellent, *slit*. Without the midline chemo-attractant NETRIN, most glands in a *netrin* mutant are repelled by SLIT and migrate laterally, away from the CNS midline. Conversely, in the absence of midline SLIT or its receptors, ROBO and ROBO2, most glands are attracted by NETRIN and other attractants at the midline and migrate medially, toward the CNS (Fig. 7).

Comparison of the *fra*- and *netrin*-mutant phenotypes suggests that, in addition to FRA, an unknown netrin receptor functions during salivary gland migration. Although the null, *fra*-mutant phenotype closely resembles that of the *netrin* null, it is much less penetrant (6% compared to 23%). UNC-5, the only other known NETRIN receptor, is an unlikely candidate; it is excluded from wild-type salivary gland cells and acts as a repulsive NETRIN receptor. Since vertebrate studies have shown that integrins can function as NETRIN receptors during pancreatic cell migration, and since salivary glands express the αPS1 integrin MEW, we tested whether MEW serves as the second NETRIN receptor during salivary gland migration. However, *mew*-mutant salivary glands curve medially or laterally with equal probability, suggesting that attractive and repulsive guidance mechanisms are not affected. Instead, in the absence of integrins, the gland appears to lose adhesion and move away from the visceral mesoderm, without lateral or medial bias (personal observation). Although MEW most likely does not serve as a NETRIN receptor during gland migration, salivary glands do become more sensitive to the levels of NETRIN in the absence of MEW. Decreasing the dose of *fra* or overexpressing *fra* in the gland in a mew mutant background biases the direction in which the gland migrates away from the visceral mesoderm. Therefore, in the absence of integrins, the glands appear to become less attached to their substrate (visceral mesoderm) and more responsive to midline guidance cues. Because of the big effects of *fra* in this *mew*-mutant background, these experiments strongly reinforce the conclusion that FRA does act as a NETRIN receptor in the salivary glands.

Since nearly all *slit*-mutant embryos have medially oriented glands, SLIT appears to be either the strongest or the only midline repellent involved in gland guidance. In contrast, only a quarter of *netrin*-mutant embryos have gland guidance defects, suggesting that other chemo-attractants may act redundantly with NETRIN during salivary gland migration. Indeed, in *netrin slit* double mutants, many salivary glands migrate toward the CNS as though they continue to be attracted by a residual guidance cue(s) expressed in the midline. The nature of this additional chemo-attractant has yet to be identified.

The similarities between axon and salivary gland guidance are remarkable considering the many physical and functional differences between nerve and epithelial cells. In addition to utilizing at least two of the same guidance cues during their migration, the systems are equally sensitive to the levels of these cues. Axon and salivary gland migrations are both greatly affected in *slit* mutants and only slightly in *netrin* mutants. Therefore, SLIT acts as a very powerful repellent while other attractants act redundantly with NETRIN during both migrations. Also, only the leading tip of the migrating axon produces cell extensions that respond to cues and lead the axon to its proper destination. This also appears to be the case during salivary gland migration. Although the presence of cell extensions primarily at the leading distal tip of the gland has not been shown, guidance cue mutant and misexpression experiments reveal that only the distal part of the glands becomes misguided, suggesting that the distal tip leads the entire gland on its path. Whether all components of the *slit* and *netrin* signaling pathways are conserved between the two systems will also be interesting to determine. While very little is known about the components of the NETRIN pathway, at least one component of the SLIT pathway, *commissureless*, which down-regulates ROBO receptors in migrating axons, appears not to be exploited during salivary gland migration. In *commissureless* mutants, ROBO is ectopically expressed by axons resulting in their abnormal repulsion by midline

SLIT, but salivary gland migration is unaffected. Testing whether molecules that act downstream of ROBO during axon migration, such as ABELSON tyrosine kinase and its substrate ENABLED, function during salivary gland migration will help clarify the distinctions between SLIT signaling in axons and salivary glands.

A further intriguing outcome of this investigation is that while *netrin* and *slit* are important to keep the gland moving parallel to the CNS, neither one guides the glands posteriorly. Perhaps in addition to *netrin* and *slit*, a third cue is involved in attracting the gland toward the posterior. The presence of a posterior guidance cue could explain the glands' ability to migrate posteriorly even in a *slit netrin* double mutant. Furthermore, during a possible third phase of salivary gland migration, the gland detaches from the visceral mesoderm and appears to become closely associated with adjacent tissues, such as fat body and somatic muscle. Whether repulsion from the visceral mesoderm and/or attraction by the neighboring tissues brings about this association is an interesting matter left for exploration. Perhaps other known axon guidance cues will function during these unresolved parts of salivary gland migration.

Interestingly, all four major families of axon guidance molecules, the netrins, slits, ephrins, and semaphorins, have been shown to function in vertebrate, non-neural tissues. For example, SLIT-2 can inhibit leukocyte migration towards sources of attractive chemokines (Wu et al., 2001), and NETRIN-1 can guide endothelial cells (Park et al., 2004). Similarly, SEMAPHORINS *3a1* and *3a2* serve as chemo-repellents to inhibit migration of major vessels in the zebrafish circulatory system (Torres-Vazquez et al., 2004) and EPHRIN-A5 repels muscle precursor cells during their migration to their limb target in avian limb muscle development (Swartz et al., 2001). Despite the multiple roles of most axon guidance cues in vertebrates, in *Drosophila* only SLIT has been previously shown to function outside of the nervous system. Besides guiding axons, SLIT also guides glial, tracheal, and somatic mesodermal cells (Englund et al., 2002; Kinrade et al., 2001; Kramer et al., 2001). We identified an additional, non-neural role for SLIT by demonstrating its ability and necessity to guide salivary glands. In addition, we are the first to show a role for *Drosophila* NETRIN beyond axon guidance. These results illustrate that irrespective of the tissue shape, size, or cell type, similar guidance mechanisms can be utilized in widely varying developing systems.

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